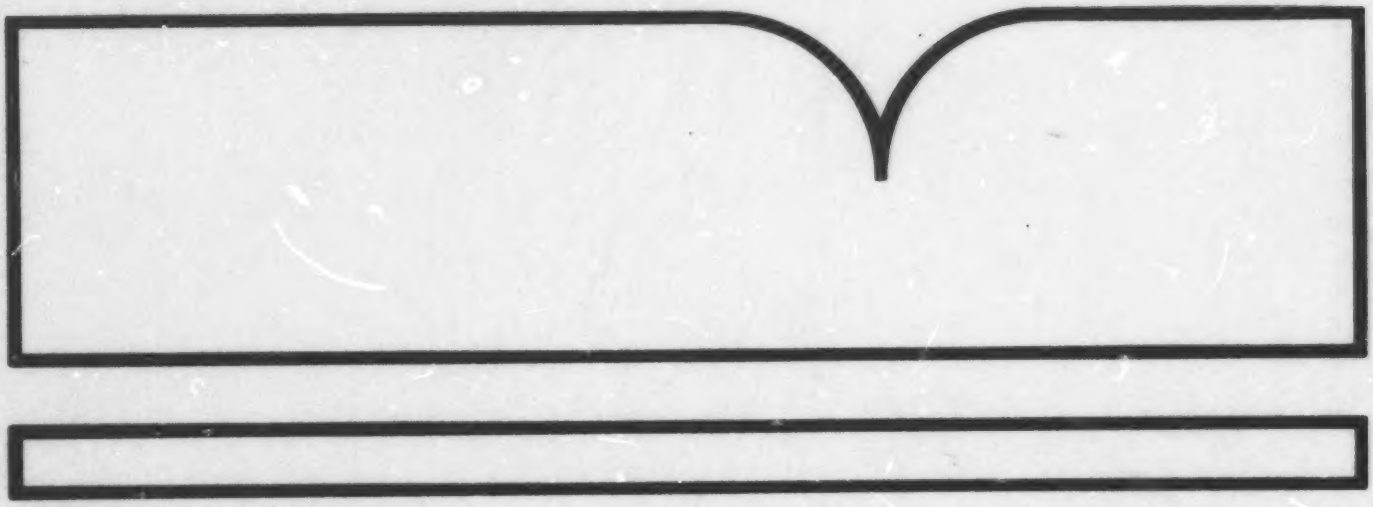
PB86-137072

Hypochlorite-Serum Reaction Products Inhibit Porcine Vascular Endothelial Cell Growth in Culture

(U.S.) Health Effects Research Lab.
Cincinnati, OH

1985



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1. REPORT NO. EPA/600/J-85/242	2.	3. R PB86-137072
4. TITLE AND SUBTITLE HYPOCHLORITE-SERUM REACTION PRODUCTS INHIBIT PORCINE VASCULAR ENDOTHELIAL CELL GROWTH IN CULTURE (Journal Version)		5. REPORT DATE 1985 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) Linda R. Papa and Paul T. McCauley		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Toxicology and Microbiology Division, HERL/ORD/USEPA, Cincinnati, OH 45268		10. PROGRAM ELEMENT NO. ABMC1A 11. CONTRACT/GRANT NO. In-house
12. SPONSORING AGENCY NAME AND ADDRESS Health Effects Research Laboratory Office of Research and Development U.S. Environmental Protection Agency Research Triangle Park, North Carolina 27711		13. TYPE OF REPORT AND PERIOD COVERED Journal Article 14. SPONSORING AGENCY CODE EPA-600/11

15. SUPPLEMENTARY NOTES
P.O. Linda R. Papa TOTB/TMD/HERL/USEPA (CI)
Citation: Artery 13(1):50-60 (1985) September

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17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

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NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

18. DISTRIBUTION STATEMENT Release to public	19. SECURITY CLASS (This Report) unclassified	21. N AGES 13
	20. SECURITY CLASS (This page) unclassified	22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS

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ARTERY 13(1):50-60 (1985)

EPA/600/J-85/242
JOURNAL ARTICLE

HYPOCHLORITE-SERUM REACTION PRODUCTS INHIBIT PORCINE VASCULAR ENDOTHELIAL CELL GROWTH IN CULTURE

Linda R. Papa and Paul T. McCauley
U.S. Environmental Protection Agency, Cincinnati, Ohio 45268

Key words: Vascular endothelium, chlorination, sodium hypochlorite

ABSTRACT In vitro toxicity studies were initiated in order to determine if chlorination affects vascular endothelial cells. Twelfth to twentieth passage porcine aortic vascular endothelial cells (PAE) were grown to confluency and replated in the presence of complete media (Eagle's minimum essential media supplemented with 20% fetal bovine serum) which had been preincubated for 30 minutes with 15.0 mg/liter chlorine. During a 72-hour exposure period, control PAE cells grew to confluency, an increase of approximately 9 fold in the number of cells/plate. Those cells exposed to media preincubated with 15.0 mg/liter chlorine derived from sodium hypochlorite increased only 6 fold. There was no sign of cell killing, but an apparent inhibition of cell division. No effect was seen when either the amino acid or vitamin component of the complete media was reacted at the 15.0 mg dose level. However, when the serum component was preincubated with 15.0 mg chlorine/liter as sodium hypochlorite, an inhibition in growth rate similar to the complete media occurred.

INTRODUCTION

Previous reports have indicated a relationship between sodium hypochlorite and mutagenesis.[1] Other studies have linked sodium hypochlorite with inhibition and modification of DNA and DNA-polymerase in bacteria.[2,3] More recently, studies have been conducted which show the formation of chlorination products following disinfection of drinking water with free chlorine.[4] Similarly, the reaction of chlorine with amino acids, fatty acids, fatty acid esters and lipids have been reported[5,6] In vivo studies

have shown the formation of chlorinated hydrocarbons in the gut and plasma of rats dosed with sodium hypochlorite.[7] Because of these findings, an in vitro assay was developed to look at the effects of chlorination on mammalian cell growth and proliferation. Since, the arterial endothelium is vital in providing a selective barrier between the plasma and the underlying vascular wall, it was selected as a possible target organ of chlorination products.[8]

METHODS AND MATERIALS

Cell Culture

Pig aortic endothelial cells (PAE) were established by collagnase digestion of the intimal layer of the thoracic aorta of fourteen day weanling pigs.[9] The cells were originally grown in Eagle's MEM supplemented with 20% fetal bovine serum (FBS), 2 mM glutamine, 50 units/ml penicillin, 50 ug/ml streptomycin (GIBCO, Grand Island, NY) and 100 ng/ml fibroblast growth factor (Collaborative Research, Waltham, MA). These cells exhibit a compact, cuboidal shape and form a contact-inhibited, cobblestone monolayer at growth confluence. Detection of factor VIII antigen by indirect immunofluorescence was used as an additional criterion to establish the endothelial identity of these cells.[10,11] Cells were subcultured and several vials frozen in liquid N_2 at early passage in 10% DMSO.

Sample Preparation

A NaOCl stock solution containing 5% active chlorine (Red Bird Service, Metamora, IN) was diluted in sterile deionized water just prior to dosing. The final concentration of chlorine was 0.5 ug/ul. The test solution was added to either complete media or individual components of the media (serum, amino acids, and/or vitamins) and sealed airtight in 50 ml centrifuge tubes. Control solutions containing an equal volume of solvent, free of test agent, were also prepared. All solutions were allowed to react for 30 minutes at 37°C, after which tubes were opened and a gentle, steady stream of 95% O_2:

5% CO_2, was bubbled through the solutions for 30 minutes at room temperature. Reacted solutions were then diluted with complete media to give a final concentration equivalent to 15 ppm HOCl/OCL reaction products in the medium. In samples where a dilution factor might have altered the osmolarity of the final growth media, a 2x MEM base media was used and diluted to the appropriate final concentration with the addition of the NaOCl reacted solution. A 5 ml aliquiot was then added to duplicate flasks of each reaction group. The flasks were sealed airtight and incubated at 37°C under a 95% O_2: 5% CO_2 atmosphere. Residual Cl^- determinations were conducted immediately following incubation and/or aeration of solutions by mercuric nitrate titration.[12]

Assay System

PAE cells were plated into 25 cm^2 culture flasks (Flacon, Oxnard, CA) at a density of 1.0×10^4 cells/cm^2 in a volume of 5 ml complete media Eagle's MEM supplemented with 20% FBS, 2 mM glutamine and 1% pen-strep (GIBCO, Grand Island, NY). After a 24-hour attachment and growth period, the media was aspirated off and fresh media containing the equivalent of 15 ppm HOCl/OCL-reaction products was added to the culture flasks. Flasks were sealed airtight and incubated in 95% O_2: 5 CO_2 at 37°C. Cell counts were performed at 0, 24, 48, 72-hour intervals after initial dosing, using a ½F Coulter counter. Cell growth was measured as the ability of the cells to proliferate and reach confluency after single or multiple exposures to the various HOCl/OCL-reaction products and plotted as the increase in mean cell number against time. Statistical evaluations were performed by an analysis of variance of regression co-efficients. A significant F ratio indicated that the slopes or growth rates differ between groups. P values less than or equal to .05 were considered significant.

RESULTS

Porcine aortic endothelial (PAE) cells cultured in minimal essential media (MEM) plus 20% fetal bovine serum grew from a density of 2.2×10^5

cells/pl to 1.9 x 10^6 cells/pl over a 72-hour period (Figure 1). In Figure 2 the normal cobblestone morphology of cultured endothelial cells is demonstrated[10]. When the culture media including fetal bovine serum also contained 15 ppm sodium hypochlorite (NaOCl) the final density of PAE cells was only 1.3 x 10^6 cells/pl or 73% of normal. Figure 3 demonstrates, however, that HOCl/OCL treated cells retained their normal cobblestone appearance but there were fewer cells. Light microscopy observations made during the growth phase of the cultures indicated no unusual degree of cell killing in NaOCl treated cultures. Just prior to introducing the NaOCl reacted media to PAE cells, the media was assayed for unreacted chlorine. The chlorine concentration was below the limits of sensitivity in culture media (0.9 ppm).

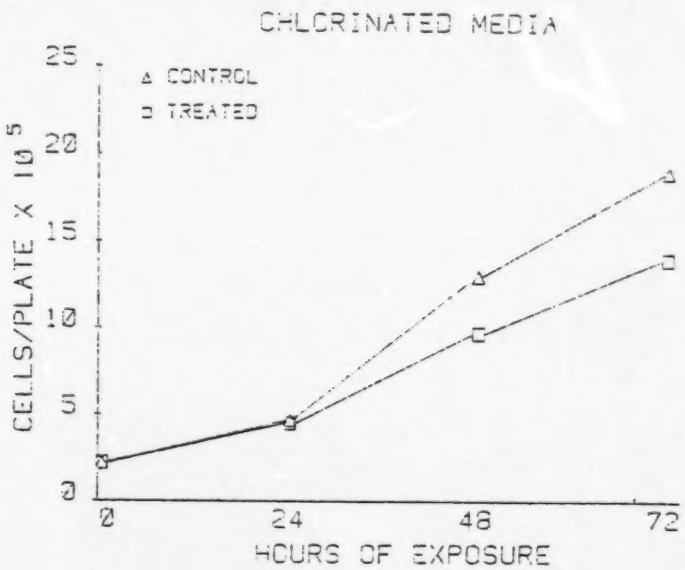

FIGURE 1

Growth of PAE cells (passage 21) exposed to medium containing 15 ppm HOCl/OCL reaction products (□--□) and control medium (Δ--Δ). Replicate cultures were incubated at 37°C following a single exposure to NaOCl. At the indicated times, the cells from duplicate flasks were electronically counted. Rate of cell growth is expressed as the increase in mean cell number over the time in culture. F ratio = 6.168; $P < .01$.

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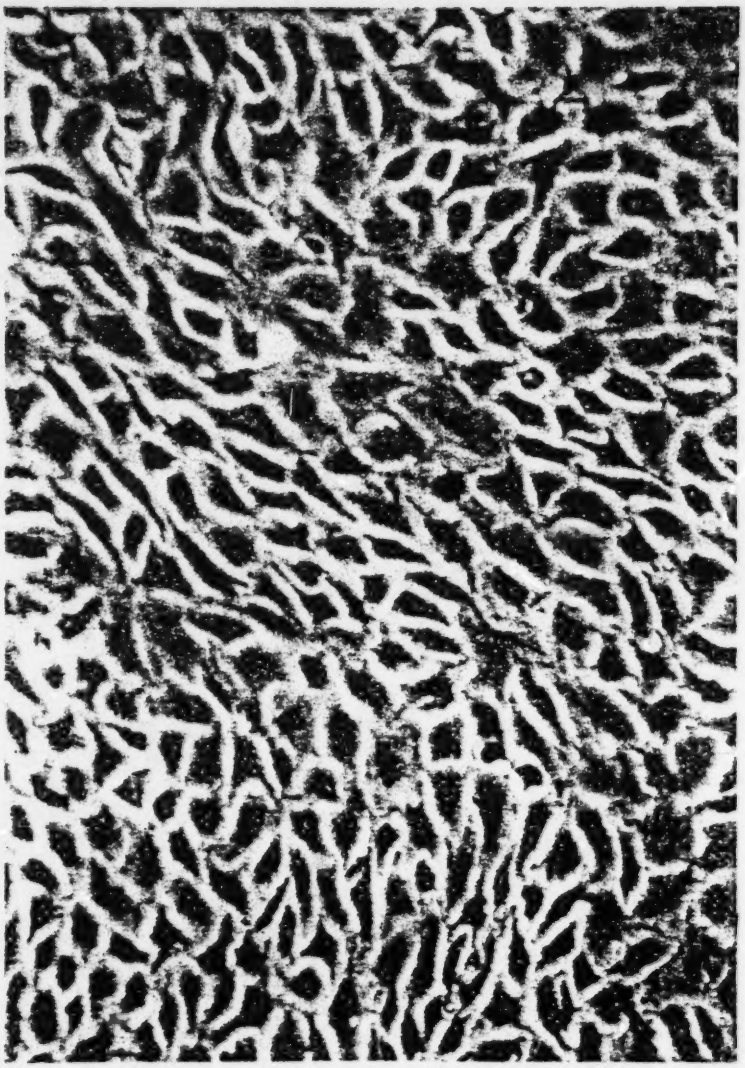

FIGURE 2

Untreated PAE cells (passage 23) showing a tightly packed near confluent monolayer of endothelial cells 72 hours after the initial plating and recovery period. Cells display a homogeneous cobblestone morphology typical of endothelial cells in culture. Cells have one prominent nucleus, two to several dark nucleoli and well defined cell borders.

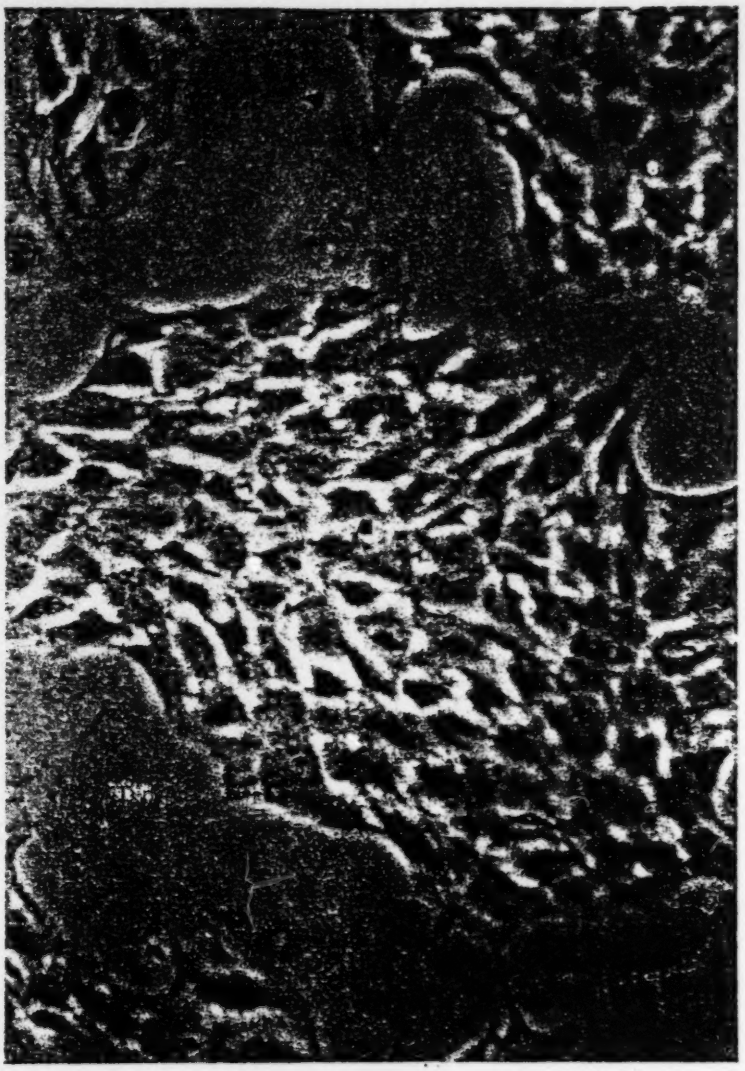

FIGURE 3

PAE cells (passage 23) which have been exposed for 72 hours to medium containing 15 ppm HOCl/OCL. These cells also exhibit the typical morphologial pattern of endothelial cells in culture and are identical in appearance to control cells (Figure 2). However, the cells do not from a confluent monolayer but rather have form isolated distinct colonies covering approximately 70% of the surface area of the flask.

Figure 4 depicts PAE cell densities measured at 0, 24, 48, and 72 hours after incubation in control and 15 ppm NaOCl treated culture media which had been aerated 30 minutes with 95% O_2: 5% CO_2 prior to cell culture use. Also plotted are cell densities recorded from PAE cells grown in identical media which had been held 30 minutes in sealed incubation flasks rather than aerated. There was no material difference between cell densities of aerated and non-aerated controls ($p > .5$). Similiarly, NaOCl media grown PAE cells demonstrated no material sensitivity to aeration. The major differences seen were between control and NaOCl treated cells ($p < .01$).

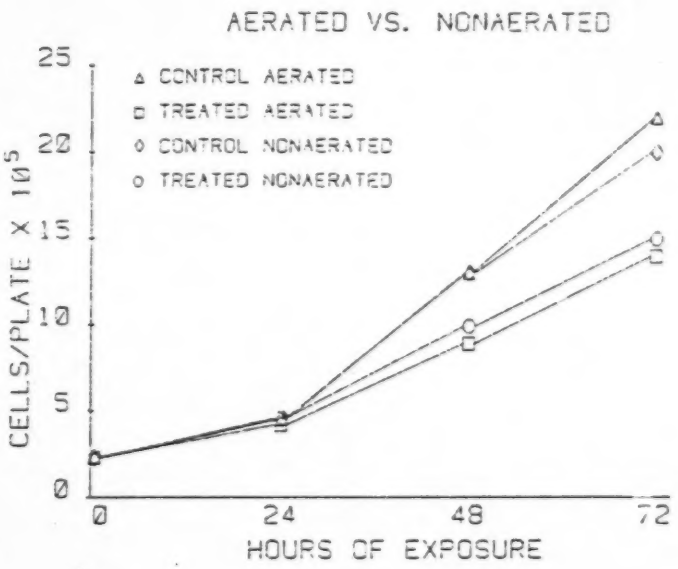

FIGURE 4

The growth rate of PAE cells following exposure to aerated medium 15 ppm NaOCl-reacted and control medium. Duplicate tubes of control and NaOCl reacted medium were incubated for 30 minutes at 37°C 95% O_2: 5% CO_2 after which non-aerated solutions were added immediately to the culture flasks. Aerated tubes were opened at room temperature and a steady gentle stream of 95% O_2: 5% CO_2 was bubbled through the media for 30 minutes then added to culture flasks. At the indicated time points duplicate flasks of each group were electronically counted. Results are expressed as the mean number of cells/plate over time in culture. F ratio aerated vs. non aerated = .598; $p > .5$.

Table I is a summary of the cell densities resulting from doubling the concentration of individual components of the media. When the amino acid concentration was doubled the final cell density increased over previous controls by approximately 11%. When amino acid doubling was achieved by adding NaOCl exposed amino acids to the 1x amino acid containing complete media, (final chlorine concentration in the media was calculated to be 15 ppm.) there was no decrease in cell density.

TABLE I

Chlorination of Media Components

Chlorinated Groups	Cells/Plate Control	Cells/Plate OCl Reacted	Cell Growth % of Control
Amino Acids (AA)	2.1×10^6	2.1×10^6	100.0%
Serum	1.8×10^6	1.3×10^6	72.9%
Serum & Vitamin & AA	1.8×10^6	1.4×10^6	77.7%
Vitamins	1.9×10^6	1.8×10^6	94.7%

Media components (serum, amino acids, and/or vitamins) were reacted with NaOCl for 30 minutes at 37° in an atmosphere of 5% CO_2: 95% O_2. Each chlorinated component or combination of chlorinated components was added to the complete media (MEM + 20% FBS - 2 mM glut - 1% pen-strep) yielding a final concentration of 15 ppm OCl-reaction products. Values are expressed as the mean number of cells/plate.

When fetal bovine serum was doubled, control 72-hour cell density was equivalent to the control values routinely found. However, when fetal bovine serum doubling was achieved by adding NaOCl reacted fetal bovine serum to complete media containing 20% unreacted fetal bovine serum, the final cell count was only 73% of control density (Table I).

When all the major media components except the salt mix were doubled and reacted with NaOCl, the 72-hour cell counts were 78% of similiarly

treated controls. The water soluble vitamin component doubling showed no material difference between controls and NaOCl treated groups.

DISCUSSION

Figure 1 demonstrates a decreased final PAE cell count after 72-hour incubation in NaOCl reacted media when compared to control. Light microscopy examination of cultures revealed no evidence of cell death during the incubation period. At no time did the experimental group demonstrate different morphology (i.e., rounded cells, floating cells or any deviation from normal cobblestone appearance) from controls (Figures 2 and 3). The probable explanation is a decreased growth rate in the experimental group.

NaOCl is one of the products formed during drinking water chlorination. The major concern of this paper was to determine if the source of toxicity to PAE cells seen in Figure 1 was due to NaOCl or a reaction product of NaOCl.

In order to test the hypothesis that NaOCl was not involved in the toxicity, the media was aerated for thirty minutes at 25°C. Aeration should decrease the NaOCl concentration. Consequently, if the observed decrease in growth rate was due to the parent compound at least the degree of inhibition (due to aeration of the NaOCl media) would be reduced when compared to the non-aerated media in sealed incubation flasks. However, there was no difference indicating that the inhibition was due to a reaction between the NaOCl and the media.

The possibility that NaOCl reacts with the culture media and either depletes the media nutritionally or in growth factor could explain the effect seen in Figures 1 and 4. The results in Table I indicate the decrease cell count is not the result of a media depletion. In all cases shown in Table I both control and experimental groups were incubated in a complete media not reacted with NaOCl. Component fractions were reacted and aerated and then added to the complete media. NaOCl reacted amino acids did not change the final count of PAE cells when compared to non-NaOCl reacted amino acid

controls. This indicated (1) amino acid-NaOCl derivatives do not express the growth inhibition in this system and (2) a residual chlorine concentration sufficient to cause inhibition does not survive aeration.

When any combination of fetal bovine serum and NaOCl preincubation was included in the complete media, a reduction of at least 20% is noted in the final PAE cell count (Table I). This is probably due to the reaction of NaOCl with something in fetal bovine serum which results in formation of a toxic agent which inhibits PAE cell growth. The nature of this inhibitory factor is under investigation.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Judy Stober for her review and assistance with the data analysis, Mr. Keith Kelty for his assistance with the chemical analysis and Ms. Pat Underwood for her assistance in the preparation of the manuscript.

The research described in this article has been reviewed by the Health Effects Research Laboratory and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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